Phytochemistry, 1976, Vol. 15, pp. 387–390. Pergamon Press Printed in England.

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE LINOLEATE HYDROPEROXIDE ISOMERASE FROM GRAINS OF *HORDEUM DISTICHUM**

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(*Received* 11 *March* 1975)

Key Word Index—*Hordeum distichum*; Gramineae; barley; embryo; linoleate hydroperoxide isomerase; linoleic acid.

Abstract—Linoleate hydroperoxide isomerase was mainly located in the embryos of barley grains and its activity decreased during germination. The enzyme partially purified from embryos converted 9-hydroperoxy, *trans*-10, *cis*-12-octadecadienoic acid to 9-hydroxy, 10-oxo, *cis*-12-octadecenoic acid and 13-hydroxy, 10-oxo, *trans*-11-octadecenoic acid in the ratio of *ca* 2:1.

INTRODUCTION

IN a previous report [1], we showed that barley embryos contained a lipoxygenase which converted linoleic acid to mainly (90%) 9-hydroperoxy, *trans*-10, *cis*-12-octadecadienoic acid. However, when barley grains were germinated at 20°, there was practically no accumulation of hydroperoxides in the embryos and endosperms, while the amount of free linoleic acid in the grain increased. These facts indicated that the hydroperoxides are further metabolized by a sequential enzyme system in barley grains.

A hydroperoxide isomerase which catalyzes the isomerization of an unsaturated hydroperoxide to a keto-hydroxy compound was first found in flax-seed by Zimmermann[2, 3]. The presence of a similar isomerase has been reported in corn germ [4]. In the present work, we isolated a linoleate hydroperoxide-decomposing enzyme from barley grains and have proved that the enzyme was linoleate hydroperoxide isomerase. Some properties of the isolated enzyme were also investigated.

RESULTS

Products from linoleic acid by sequential enzyme reactions

In a preliminary experiment, linoleic acid was incubated in a barley flour suspension and the products from sequential enzyme reactions were identified. TLC separation of the products from linoleic acid gave two major spots (A and B), R_f values 0·19 and 0·09 respectively, which were coloured yellow with 2,4-dinitrophenylhydrazine reagent. The two products, A and B, were then isolated by preparative-TLC. The IR spectra of the two products (as methyl esters) clearly showed ketone carbonyl absorption at 1720 cm^{-1} and secondary OH absorption near 3500 cm^{-1}, indicating that the two isolated compounds would probably be octadecenoic acids with ketone and OH groups [3, 4]. Determination of the full structure of the two compounds was performed using a purified enzyme preparation as described below.

Extraction of the linoleate hydroperoxide isomerase from barley embryos

The efficiency of extracting the isomerase from ungerminated barley embryos was examined using phosphate buffer solutions of different pH and molar concentrations. As shown in Table 1, 0·5 M Pi buffer, pH 7·5, containing 0·2% Tween 20 was most effective in extracting the isomerase from the embryos. However, since the presence of Tween 20 interfered with the ammonium sulfate precipitation of the active enzyme fraction, the extraction of the crude enzyme from the embryos was usually performed with 0·5 M Pi buffer, pH 7·5, containing no Tween 20.

Isomerase activity in germinating barley grains

As shown in Table 2, the hydroperoxide isomerase activity appeared to be mainly located in the embryos,

Table 1. Extraction of isomerase from barley embryos

Extracting medium	Activity	Extracting medium	Activity
pH 7·5		pH 7·5	
0·1 M Pi buffer	0·31*	0·5 M Pi buffer	
0·2 M Pi buffer	0·39	with Tween 20	
0·5 M Pi buffer	0·63	0%	0·64
1·0 M Pi buffer	0·42	0·1	0·92
0·5 M Pi buffer		0·2	0·98
pH 5·5	0·32	0·5	0·98
pH 6·0	0·48	1·0	0·93
pH 7·0	0·50	2·0	0·90
pH 7·5	0·61		
pH 8·0	0·60		
pH 9·0	0·55		

* Units/50 μl. (Extracts were prepared by grinding the embryos from 25 grains with 5 ml of the various extracting media, then centrifuging them at 10000 *g* for 10 min.)

* Part 2 in the series "Studies of Lipid Metabolizing Enzymes in Barley Grains". For Part 1 see

Table 2. Linoleate hydroperoxide isomerase activity in germinating barley grains

Tissue	Time after steeping 4 hr	2 days	4 days	6 days
Embryos	45·5*	39·8	19·3	8·7
Endosperms	6·0	2·4	4·4	2·9

* Units/ml of extract.

though there was some isomerase activity in the endosperms. The isomerase activity in the embryos decreased markedly during germination, as has been observed with flaxseeds by Zimmermann and Vick[3]. The embryos dissected from ungerminated grains (4 hr of steeping) were therefore used for enzyme extraction.

Purification of the isomerase from barley embryos

Embryos dissected from about 10000 grains were homogenized with 0·5 M Pi buffer, pH 7·5, then centrifuged for 20 min at 35000 *g* to obtain crude extracts. Ammonium sulfate was added to the crude extract to give 40% saturation. The precipitate was collected, dialyzed and applied to a DEAE-Sephadex A50 column that had been previously equilibrated with 0·01 M Pi buffer, pH 7·5. Elution was carried out with 500 ml of 0·5 M Pi buffer, pH 7·5, containing 0·2% Tween 20, and the isomerase active fractions were pooled. The pooled fraction was brought to 40% saturation with ammonium sulfate and the precipitates were collected. Precipitates were then applied to a Sephadex G-200 column and eluted with 0·1 M Pi buffer, pH 7·5. The fractions showing the linoleate hydroperoxide isomerase activity were pooled and stored in a refrigerator.

Specific activities and yields of the isomerase at four different stages of purification are shown in Table 3. Protein contents were assayed by the method of Lowry *et al.* [5] using bovine serum albumin as the standard. The final preparation had a specific activity of 303 units, about 22 fold that of the initial crude extracts. It contained no lipoxygenase activities, but its protein profile on the final column chromatography was not completely homogeneous. This partially purified preparation was used for the determination of the reaction products.

Table 3. Purification of the linoleate hydroperoxide isomerase from barley grains

Purification stage	Specific activity (units/mg of protein)	Total activity (units)	Yield (%)
Crude extracts	13·7	33800	100
Precipitates at 40% saturation of ammonium sulfate	23·2	24600	73
DEAE Sephadex A50	105	18500	55
Sephadex G-200	303	10100	30

Reaction products from linoleate hydroperoxide using the purified isomerase

When the linoleate hydroperoxide prepared using barley lipoxygenase [1] was incubated with the partially purified isomerase preparation, two main products, A and B, which correspond to spots A and B in the preliminary experiments were again produced. The amounts of the two products were *ca* in the ratio of 2:1 on scanning them with a densitometer after charring with 50% H_2SO_4.

Each of the two products was then isolated from the TLC plate and analyzed by IR and microchemical techniques as shown in Fig. 1. The aldehydes and acids produced from the various chemical treatments were identified by GLC comparison with authentic compounds.

Product A gave hexanoic acid and nonanedioic acid by oxidation with $KMnO_4$–$NaIO_4$. Formation of nonanoic acid from spot A on $NaIO_4$ oxidation followed by reduction with hydrogen in the presence of platinum oxide indicated that nonenoic acid and nonanoic 9-al were produced by $NaIO_4$. Reduction of spot A with $NaBH_4$ and H_2/PtO_2 followed by oxidation with $NaIO_4$ resulted in the formation of nonanal and nonanoic 9-al. The IR spectrum of A clearly showed the presence of a *cis* double bond at 3015 cm^{-1}; thus, product A was identified as 9-hydroxy, 10-oxo, *cis*-12-octadecenoic acid.

Product B produced decanedioic acid in addition to hexanoic and nonanedioic acids on $KMnO_4$–$NaIO_4$ oxidation as has been observed by Gardner[4]. No acid was formed from B on $NaIO_4$ oxidation. The IR spectrum of B showed the presence of a *trans* double bond at 973 cm^{-1} and was identical with that of the 13-hydroxy, 10-oxo, *trans*-11-octadecenoic acid described by Gardner[4].

These results show that the isolated barley enzyme was a hydroperoxide isomerase that converted the 9-hydroperoxide of linoleic acid to the two major isomers, 9-hydroxy, 10-oxo, *cis*-12-octadecenoic acid and 13-hydroxy, 10-oxo, *trans*-11-octadecenoic acid.

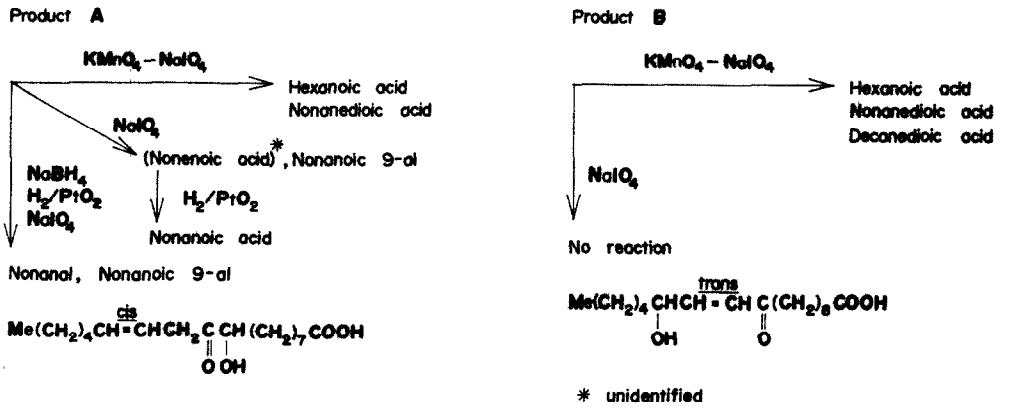

Fig. 1. Flow chart showing microchemical reactions used for identification of products A and B.

Table 4. Effect of various reagents on the activity of the linoleate hydroperoxide isomerase

Reagent	Concentration (M)	Relative activity (%)
Control	–	100
KCN	10^{-3}	95
EDTA	10^{-3}	96
ICH_2COOH	10^{-3}	100
ICH_2CONH_2	10^{-3}	90
N-Ethylmaleimide	10^{-3}	88
$Hg(MeCOO)_2$	10^{-4}	3
$CuSO_4$	10^{-4}	91
$CuSO_4$	10^{-3}	72
$CuSO_4$	10^{-2}	54

Preincubation conditions; 20° for 30 min in 0·1 M KPi buffer, pH 6·8.

Other properties of the linoleate hydroperoxide isomerase

The optimum pH of the barley linoleate hydroperoxide isomerase was *ca* 6·8, the activity being reduced to half at pH 5·5 and 9·0. The activity of the isomerase was not inhibited by 1 mM KCN, EDTA, monoiodoacetate or *N*-ethyl maleimide (Table 4). The presence of Hg^{2+} or Cu^{2+} ions at 10^{-2}–10^{-4} M was inhibitory (Table 4) to the isomerase activity, though they were not inhibitory to the barley lipoxygenase [1].

When the isomerase was dissolved in 0·1 M Pi buffer, pH 6, and heated at 45° and 60° for 60 min, the remaining activity was 65 and 0% respectively, indicating that the enzyme is heat-sensitive. When the enzyme solutions in 0·1 M buffer of different pHs were kept at 20° for 20 hr, about 90% of the original activity was retained at level over pH 7, whereas only 40% remained at pH 5.

DISCUSSION

The linoleate hydroperoxide isomerase from barley embryos was found to be similar to that from corn [4] in that it converted 9-hydroperoxy linoleic acid into 9-hydroxy, 10-oxo, *cis*-12-octadecenoic acid and 13-hydroxy, 10-oxo, *trans*-11-octadecenoic acid. The ratio of the two reaction products was *ca* 2:1 when barley flour suspensions or the partially purified enzyme preparations were used as the enzyme source. This indicates that the same isomerase is involved in the formation of the two ketol acids. No other products could be detected on TLC, even when linoleic acid was incubated for more than 60 min in barley flour suspensions. Thus, from these *in vitro* experiments, it appears that the isomerase could be the final enzyme on the linoleic acid oxidation pathway.

As to the physiological role of linoleate hydroperoxide isomerase, Veldink *et al.* [6] have suggested that, *in vivo*, the isomerase protects the seed against excessive amounts of linoleic acid hydroperoxides which may be toxic to seed germination. Jadhav *et al.* [7] have shown, however, that the ketol acids from linoleic and linolenic acids could be intermediates in the biogenesis of hexanal in the tomato fruit.

EXPERIMENTAL

Materials. A single lot of grains of two-row barley, *Hordeum distichum* cv. Chigasaki Nijo (1973 harvest) were used in all experiments. Linoleate hydroperoxides were prepared from barley lipoxygenase and linoleic acid [1], and stored in MeOH at 5–10°.

Sequential reactions in barley flour suspensions. Barley flour (5 g) and 70 mg of linoleic acid (99% pure) were homogenised in 35 ml of 0·1 M Pi buffer, pH 7. After incubation on a shaker at 25° for 60 min, the suspensions were acidified with HCl to pH 2 and extracted with $CHCl_3$–MeOH (2:1). A portion of the extract was applied to a Si gel TLC plate which was developed with petrol–Et_2O–HOAc (60:40:1). The separated compounds were detected by charring with 50% H_2SO_4 and heating for 10 min at 200°, or by spraying them with 2,4-dinitrophenyl-hydrazine reagent.

Germination, separation of tissues, and extraction of the enzyme. Dehusked barley grains were maintained at 20° for 6 days and germinating samples were taken at intervals of 2 days. The embryos and endosperms from 100 grains were dissected with a knife and each portion was extracted separately with 10 ml of 0·5 M Pi buffer, pH 7·5, containing 0·2% Tween 20. The supernatants obtained by centrifugation at 35000 *g* for 10 min were used as the crude enzyme extracts.

Enzyme assay. Linoleate hydroperoxide isomerase activity was determined at 20° by measuring the conjugated diene absorption at 234 nm with a recording spectrophotometer equipped with a 1 cm cell. Incubation mixtures (3 ml) contained linoleate hydroperoxides (initial A at 234 nm = 1) dissolved in MeOH; 1·5 ml of 0·2 M KPi buffer, pH 6·8; and an appropriate amount of enzyme soln. The activity was not affected by the presence of MeOH up to 5%. The enzyme activity was calculated from the initial rates of the decrease in A_{234} nm. One unit of linoleate hydroperoxide isomerase corresponds to a decrease in A_{234} of 1·0 per min.

Analysis of enzymatic reaction products. Linoleate hydroperoxides (17 mg) and the purified preparation of linoleate hydroperoxide isomerase (12 units) were incubated in 0·1 M KPi buffer, pH 6·8, for 30 min at 20°. The reaction mixture was acidified with HCl, extracted with $CHCl_3$–MeOH (2:1), then esterified with CH_2N_2. The methyl esters were separated by preparative-TLC on Si gel. The two main products, A and B, were removed from the plate. The following microchemical methods were used to determine the full structure of each product. (i) Permanganate-periodate oxidation was carried out according to ref. [8]. Oxidation fragments were esterified with CH_2N_2 and analyzed by GLC. (ii) Periodate oxidation was carried out in 90% HOAc according to ref. [9]. Et_2O extracts from the periodate oxidation mixtures were divided into two portions. One portion was carefully evaporated, then dissolved in Et_2O for GLC analysis of volatile aldehydes. The other was esterified with CH_2N_2 for GLC analysis of carboxylic acids. (iii) Hydrogenation with $NaBH_4$ or H_2/PtO_2 was carried out according to ref. [10]. (iv) GLC analysis of the oxidation products was carried out using a 2 m column packed with 10% (w/w) diethylene glycol succinate on Neopack 1A using FID. The column temp. was programmed from 70° to 180° at 5°/min and the N_2 flow rate was 50 ml/min. At 180°, the temp. was held isothermally until all the material had eluted. R_t's were determined for the following compounds: Me hexanoate (3·5 min); Me nonanoate (9·5 min); diMe nonanedioate (24 min); diMe decanedioate (26 min) and nonanal (7 min). Me nonanoate 9-al was synthesized by the periodate oxidation of 9,10-dihydroxystearic acid followed by esterification. When this compound was analyzed by GLC isothermally at 180°, the R_t was 4 min.

Acknowledgements—We thank Mr. Y. Takahashi, President of Asahi Breweries Ltd., for permission to publish this study.

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